

**SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)**

**Assessment of factors influencing osteoprotegerin levels and  
standardization of 25-hydroxyvitamin D data  
from the HunMen study**

**by Éva Jakab**

**Supervisor: Harjit Pal Bhattoa, MD, PhD**



**UNIVERSITY OF DEBRECEN  
KÁLMÁN LAKI DOCTORAL SCHOOL**

**DEBRECEN, 2019**

**Assessment of factors influencing osteoprotegerin levels and  
standardization of 25-hydroxyvitamin D data  
from the HunMen study**

by Éva Jakab, Clinical Laboratory Scientist MSc, Clinical Biochemist

Supervisor: Harjit Pal Bhattoa, MD, PhD

Kálmán Laki Doctoral School, University of Debrecen

**Head of the Examination Committee:**

Prof. György Balla, MD, PhD, DSc, member of HAS

**Members of the Examination Committee:**

Ágnes Simon, MD, PhD

Prof. Emese Kiss, MD, PhD, DSc

The Examination takes place at the Library of Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, at 11:00 AM; on 22 October, 2019.

**Head of the Defense Committee:**

Prof. György Balla, MD, PhD, DSc, member of HAS

**Reviewers:**

Prof. Sándor Szántó, MD, PhD, DSc

Prof. Attila Patócs, MD, PhD, DSc

**Members of the Defense Committee:**

Ágnes Simon, MD, PhD

Prof. Emese Kiss, MD, PhD, DSc

The PhD Defense takes place at the Lecture Hall of Building “A”, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 02:00 PM; on 22 October, 2019.

## INTRODUCTION

Osteoprotegerin (OPG) and receptor activator of nuclear factor- $\kappa$ B ligand (RANKL), two members of the tumor necrosis factor receptor super family, are essential in the regulation of bone resorption. In addition to being a key regulator of osteoclastogenesis, the OPG/RANKL/RANK system is reported as being a significant mediator between factors such as estradiol, testosterone, parathyroid hormone, vitamin D, and bone turnover. Data on OPG and RANKL levels in healthy men is accumulating. Nonetheless, based on published data, it may well be generalized that the association between OPG/RANKL and its predictors is lacking consensus. Furthermore, the association of cystatin C with OPG and RANKL has only been limitedly investigated in the healthy, where no significant correlation was found. According to literature data, the role of cystatin C in the regulation of bone metabolism is largely expected to be clarified.

Nonetheless, in-vitro studies have implied that cystatin C is a cysteine proteinase inhibitor that decreases osteoclastogenesis by interfering at a late stage of pre-osteoclast differentiation. Additionally, cystatin C possesses the advantage of being independent of gender, muscle mass and age; and consequently may qualify better than creatinine as a surrogate marker of renal function in investigating the role of decreased protein clearance as a potential cause of age-related OPG elevation.

We determined serum OPG and sRANKL levels and examined its relationship with age, cystatin C, biochemical markers of bone turnover (osteocalcin, C-terminal telopeptides of type-I collagen, procollagen type 1 amino-terminal propeptide, 25-hydroxyvitamin D, parathyroid hormone), sex hormones (total  $17\beta$ -estradiol, total testosterone) and bone mineral density in randomly selected ambulatory men belonging to the HunMen cohort.

The role of vitamin D in regulating circulating levels of calcium and phosphorus to ensure normal bone mineralization is well known. Emerging evidence correlates insufficient levels of vitamin D to an increased risk of developing non-skeletal pathologies: cardiovascular diseases, hypertension, cancer, diabetes, multiple sclerosis, rheumatoid arthritis, infectious diseases. Maintaining sufficient vitamin D levels is therefore key to maintain good general health. Low 25-hydroxyvitamin D (25OHD) levels are associated with poor skeletal and extra-skeletal health. Trials of vitamin D supplementation and the Institute of Medicine (IOM) systematic review advocate maintaining the serum 25OHD concentration between 50 and 100 nmol/L, whereas the Endocrine Society and several others suggest that a minimum level of 75 nmol/L is necessary in older adults to minimize the risk of falls and

fracture. The optimal serum 25OHD concentrations for extra-skeletal health are evolving but appear to be above 75 nmol/L.

As such, development of robust healthcare policy to improve vitamin D sufficiency is critical and is primarily based on vitamin D data achieved from measurement of total 25OHD. The gold standard method for vitamin D metabolite testing is the liquid chromatography tandem-mass spectrometry (LC-MS/MS) technique. Nonetheless, vitamin D sufficiency is estimated by determining 25OHD concentrations and although there is abundance of data on vitamin D levels, standardization of 25OHD values is still a challenge. Lately, the Vitamin D Standardization Program (VDSP) has delineated protocols for standardizing existing 25OHD data from national surveys around the world. In short, the VDSP suggests identifying a batch of samples from the sample pool used primarily to determine 25OHD in the given survey, get the 25OHD measurements done in the selected batch using the National Institute for Standards and Technology (NIST) and Ghent University reference measurement procedures (RMP) and use the results attained, as such, to correct the originally measured 25OHD values of the sample pool. This approach is well defined but the reference measurement procedure requires special equipment and expertise that are available mainly at specialized laboratories, furthermore, the financial aspect may also limit its utility. Additionally, from a practical point of view, the VDSP approach does not suffice in allowing the correction of 25OHD results generated on a daily basis in routine diagnostic laboratories.

The Vitamin D External Quality Assessment Scheme (DEQAS) survey is an international external quality assurance (EQA) survey with more than a thousand participating laboratories and encompassing all currently available commercial platforms for 25OHD measurements. In each DEQAS survey (four per year), five samples are dispatched by regular post at ambient temperature for evaluation by the participating laboratory. In a given survey report, percentage bias from All Laboratory Trimmed Mean (ALTM) is reported for the total 25OHD measurement results of the five samples measured by the participating laboratory. As of April 2013, DEQAS also reports values assigned for each sample by the RMP of the NIST, allowing participating laboratories to evaluate the accuracy of their own results by comparing them to the NIST target values.

We used DEQAS data to formulate a master formula in order to standardize 25OHD values from the HunMen cohort.

## **AIMS OF THE THESIS**

Our study is based on the previously published HunMen study, which involved randomly selected healthy men over 50 years of age from Debrecen, from September 2009 to September 2010 to characterize the prevalence and seasonal variation of hypovitaminosis D and its relationship to bone metabolism.

### **Assessment of factors influencing osteoprotegerin levels**

In the first phase of our work we aimed to evaluate serum osteoprotegerin and soluble receptor activator of nuclear factor  $\kappa$ B ligand levels and examined its relationship with age, cystatin C, biochemical markers of bone turnover (osteocalcin, C-terminal telopeptides of type-I collagen, procollagen type 1 amino-terminal propeptide, 25-hydroxyvitamin D, parathyroid hormone), sex hormones (total  $17\beta$ -estradiol, total testosterone) and bone mineral density in randomly selected ambulatory men belonging to the HunMen cohort.

### **Standardizing 25-hydroxyvitamin D data from the HunMen cohort**

In the second phase of our work we aimed to standardize total 25OHD values of the HunMen cohort. Since there was a switch in methodology at our routine laboratory from high-pressure liquid chromatography (HPLC) to chemiluminescence immunoassay (CLIA), the original 25OHD values were reanalysed using DiaSorin LIAISON<sup>®</sup> 25OHD total CLIA methodology. We applied our approach for standardization by correction of the measured 25OHD values using the linear regression equation derived from the analysis of relationship between total 25OHD values measured by the current methodology used by the laboratory and the NIST total target values reported by the DEQAS for all 5 of the DEQAS samples in a given survey.

## **MATERIALS AND METHODS**

### **Study subjects**

The fasting serum samples (n = 194) for OPG and soluble RANKL determinations belonged to participants in the HunMen cross-sectional, observational study. Briefly the HunMen study was a local initiative to evaluate the bone health of randomly selected healthy men. Men residing in Debrecen, Hungary were invited to participate in the study from September 2009 to September 2010. During this period, a total of 229 randomly selected volunteers agreed to participate. Permission was received from the local ethics committee, after conception of the study design, and study procedures were carried out following receipt of written informed consent, volunteers not confirming to the inclusion and/or confirming to the exclusion criteria (n=3) were excluded from the final statistical analysis. Public attention to the initiative was achieved by placing posters summarizing the main aspects of the survey at general medical dispensaries. Detailed medical history was collected, and blood sampling was done after overnight fasting. Inclusion criteria were over 50 years of age, male, ambulatory, community dwelling, and generally regarded as healthy. Exclusion criteria were known prevalent metabolic bone disease, liver or renal disease (values  $\geq 2 \times$  upper limit of normal (ULN) for total bilirubin, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transpeptidase, alkaline phosphatase, lactate dehydrogenase, cholinesterase, urea, creatinine, and uric acid resulted in exclusion), and use of medication influencing bone metabolism (excluding calcium and vitamin D supplementation).

### **Study procedures**

Serum total OPG and sRANKL were measured using enzyme immunoassays (Biomedica Gruppe, Biomedica Medizinprodukte GmbH & Co KG, Wein, Austria). The OPG assay, that detects monomeric dimeric and ligand bound OPG, uses a monoclonal mouse anti-OPG antibody as capture antibody and a biotin-labeled goat polyclonal antibody for detection. The sRANKL assay, that detects soluble, uncomplexed human RANKL, uses human recombinant OPG for capture and a biotin-labeled goat polyclonal antibody for detection.

Serum 25-hydroxyvitamin D was analyzed by the automated LIAISON<sup>®</sup> DiaSorin total 25OHD chemiluminescence immunoassay (DiaSorin Inc., Stillwater, MN, USA).

The LIAISON<sup>®</sup> 25OHD-Vitamin assay is a direct competitive chemiluminescence immunoassay for quantitative determination of total 25OHD in serum. During the first

incubation, 25OHD is dissociated from its binding protein and binds to the specific antibody on the solid phase. After 10 minutes the tracer, (vitamin D linked to an isoluminol derivative) is added. After a second 10 minute incubation, the unbound material is removed with a wash cycle. Subsequently, the starter reagents are added to initiate a flash chemiluminescent reaction. The light signal is measured by photomultiplier as relative light units (RLU) and is inversely proportional to the concentration of 25OHD present in calibrators, controls, or samples.

Serum 17 $\beta$ -estradiol (E2), testosterone (T), parathyroid hormone (PTH), osteocalcin (OC), C-terminal telopeptides of type-I collagen (CTx), procollagen type 1 amino-terminal propeptide (PINP), sex hormone binding globulin (SHBG) were measured using electrochemiluminescence immunoassay (ECLIA; Roche MODULAR E170 Diagnostics GmbH, Mannheim, Germany).

The Elecsys Estradiol III assay employs a competitive test principle using two monoclonal antibodies specifically directed against 17 $\beta$ -estradiol. Endogenous estradiol released from the sample by mestrolone competes with the added estradiol derivative labeled with a ruthenium complex for the binding sites on the biotinylated antibody.

The Elecsys Testosterone II assay is based on a competitive test principle using a high affinity monoclonal antibody (sheep) specifically directed against testosterone. Endogenous testosterone released from the sample by 2-bromoestradiol competes with the added testosterone derivative labeled with a ruthenium complex for the binding sites on the biotinylated antibody.

The Elecsys SHBG assay is an one-step sandwich immunoassay, based on the use of two mouse monoclonal antibodies and on the streptavidin-biotin technology. The first antibody is biotinylated and used as a capture antibody that binds to streptavidin-coated microparticles. The second antibody, covalently linked with ruthenium, is used for detection. The free estradiol index (FEI) and free testosterone index (FTI) was calculated as the total 17 $\beta$ -estradiol to SHBG ratio and total testosterone to SHBG ratio, respectively.

The Elecsys assay for determining intact PTH employs a sandwich test principle in which a biotinylated monoclonal antibody reacts with the N-terminal fragment (1-37) and a monoclonal antibody labeled with a ruthenium complex reacts with the C-terminal fragment (38-84).

The Elecsys N-MID osteocalcin assay utilizes two monoclonal antibodies, one specific for epitopes on the N-MID OC fragment and the other specific for the N terminal OC fragment, and therefore detects the intact OC as well as the N-MID fragment.

Beta-CrossLaps assay a 2-site immunometric (sandwich) assay using electrochemiluminescence detection. Patient specimen, biotinylated monoclonal beta-CrossLaps-specific antibody, and monoclonal beta-CrossLaps-specific antibody labeled with ruthenium react to form a complex. Streptavidin-coated microparticles act as the solid phase to which the complex binds. Voltage is applied to the electrode, inducing a chemiluminescent emission from the ruthenium, which is then measured against a calibration curve to determine the amount of beta-CrossLaps in the patient specimen. This assay is specific for crosslinked isomerized type I collagen fragments, independent of the nature of the crosslink. The assay specificity is guaranteed through the use of 2 monoclonal antibodies, each recognizing linear beta-8AA octapeptides. The assay, therefore, quantifies all type I collagen degradation fragments that contain the isomerized octapeptide beta-8AA twice (beta-CTX).

The Elecsys procollagen type 1 amino-terminal propeptide assay uses the sandwich principle in which P1NP-specific, biotinylated monoclonal antibodies reacts with the P1NP-specific, ruthenium-complexed monoclonal antibodies.

Cystatin C was measured with BN ProSpec analyzer using particle-enhanced nephelometric immunoassay (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany). Polystyrene particles coated with specific antibodies to human cystatin C are aggregated when mixed with samples containing human cystatin C. These aggregates scatter a beam of light passed through the sample. The intensity of the scattered light is proportional to the concentration of the respective protein in the sample. The result is evaluated by comparison with a standard of known concentration.

Serum creatinine was measured using the Creatinine Jaffé 2nd generation (compensated) test on the cobas c 111 system (Roche Diagnostics GmbH, Mannheim, Germany). The 4-variable modification of diet in renal disease (MDRD) study equation was used for calculating eGFR. Additionally, eGFR was also calculated using the chronic kidney disease epidemiology collaboration (CKD-EPI) cystatin C and CKD-EPI creatinine-cystatin C equations.

### **Characterization of DEQAS samples**



The DEQAS survey is an international external quality assurance survey with more than a thousand participating laboratories and encompassing all currently available commercial platforms for 25OHD measurements. In each DEQAS survey (four per year), five samples are dispatched by regular post at ambient temperature for evaluation by the participating laboratory. The results are returned online and, after the submission deadline, a report is made available that can be downloaded from the DEQAS website. In a given survey report, percentage bias from ALTM is reported for the total 25OHD measurement results of the five samples measured by the participating laboratory. Submitted results are ranked in ascending order and the highest 5% and the lowest 5% (10% in total) are removed. The arithmetic mean of the remaining results is the ALTM. The ALTM has been reported to be very similar to the NIST target value measured for each sample using the NIST RMP. As of April 2013, DEQAS also reports values assigned for each sample by the RMP of the NIST, allowing participating laboratories to evaluate the accuracy of their own results by comparing them to the NIST target values. As such, the target value may substitute the value as was to be obtained by analysis with the reference procedure. In addition to ‘Total 25OHD’ (25OHD<sub>3</sub>+25OHD<sub>2</sub>), DEQAS also conveys the NIST values for the individual metabolites, i.e., 25OHD<sub>3</sub>, 25OHD<sub>2</sub> and 3-epi-25OHD<sub>3</sub>.

### **Examination of DEQAS and HunMen samples**

In 2009–2010, the samples from the HunMen cohort were analyzed by HPLC using a Jasco HPLC system (Jasco, Tokyo, Japan) and Bio-Rad reagent kit (Bio-Rad Laboratories, Hercules, CA, USA). For the determination of 25OHD<sub>3</sub> and 25OHD<sub>2</sub> samples have to be prepared. The sample is purified by precipitation reagents from the proteins and interfering analytes, followed by separation of the metabolites (25OHD<sub>3</sub>, 25OHD<sub>2</sub>, internal standard) on reverse silica column. Quantitative determination of the separated 25OHD by UV absorption detection at 265 nm.

In our routine diagnostic laboratory, due to the ever-increasing volume of the samples processed for 25OHD determination, we switched to the automated DiaSorin LIAISON<sup>®</sup> CLIA platform in June of 2014.

We reanalyzed samples belonging to the HunMen cohort using the automated DiaSorin LIAISON<sup>®</sup> total 25OHD CLIA. Our laboratory participates in DEQAS and the timing of reanalysis, with the automated DiaSorin LIAISON<sup>®</sup> 25OHD CLIA platform, coincided with that of the October 2015 DEQAS samples’ measurement. Identical analytic

conditions, i.e., reagents with the same LOT number, were insured for both the HunMen sample reanalysis and the DEQAS sample measurements. Finally, the HunMen 25OHD values were corrected using the linear regression equation derived from the analysis of relationship between our Liaison measured total 25OHD values and the DEQAS NIST total target values for all five of the DEQAS samples.

### **Bone density measurements**

Dual energy X-ray absorptiometry examination was performed at the University of Debrecen, Department of Obstetrics and Gynaecology, using the LUNAR Prodigy (GE-Lunar Corp., Madison, WI, USA) densitometer. BMD was measured at L1–L4 lumbar spine (LS) and femur neck (FN).

### **Statistical analysis**

The results of this study were analyzed “*post hoc*”. Descriptive statistics are presented as mean, median, range and standard deviation (SD). Kolmogorov-Smirnov test was used for the evaluation of the normality of the data. Most parameters were non normally distributed, therefore analyses were performed by Mann–Whitney U test. The Spearman’s rho was calculated for correlation analysis. Associations were tested using linear regression analysis after log transformation of not normal variables. Additionally, to reduce the confounding effect of age, the median age was used to form two subgroups.

A value of  $p < 0.05$  was considered statistically significant. All analyses were performed with the SPSS Statistics software, version 19.0 (IBM Corps. Armonk, NY, USA).

## RESULTS

### Subject characteristics

In order to reduce the confounding effect of age, using the median age of 59 years, the study population was divided into a middle-aged (those  $\leq 59$  years of age,  $n=98$ ) and an older (those  $> 59$  years of age,  $n = 96$ ) sub-group. Compared to the middle-aged (age:  $\leq 59$  years,  $n = 98$ ), older men had significantly higher serum OPG and significantly lower RANKL levels and RANKL/OPG ratios.

### Assessment of factors influencing osteoprotegerin levels

Serum OPG significantly correlated with age, cystatin C, E2 and FTI. Furthermore, Cystatin C correlated with age (Spearman's  $\rho = 0.377$ ,  $p < 0.001$ ), creatinine (Spearman's  $\rho = 0.487$ ,  $p < 0.001$ ), MDRD eGFR (Spearman's  $\rho = -0.524$ ,  $p < 0.001$ ), E2 (Spearman's  $\rho = 0.222$ ,  $p = 0.003$ ), P1NP (Spearman's  $\rho = 0.151$ ,  $p = 0.040$ ) and OPG (Spearman's  $\rho = 0.298$ ,  $p < 0.001$ ). Although OPG did not correlate with serum creatinine or MDRD eGFR, it did show a significant correlation with cystatin C-eGFR, and cystatin C and creatinine-eGFR. Furthermore, only the older men showed significant correlation between serum OPG and cystatin C ( $r = 0.322$ ,  $p = 0.002$ ) and E2 ( $r = 0.211$ ,  $p = 0.043$ ). Compared to the middle-aged (age:  $\leq 59$  years,  $n = 98$ ), older men had significantly higher serum OPG and significantly lower RANKL levels and RANKL/OPG ratios. The middle-aged individuals showed no significant correlation between serum OPG and sRANKL levels and the other studied parameters (age, BMI, LS BMD, FN BMD, PTH, 25OHD, OC, CTx, P1NP, cystatin C). The older men showed a significant correlation between serum OPG levels and cystatin C ( $\rho = 0.322$ ,  $p = 0.002$ ), and serum OPG levels and E2 ( $\rho = 0.211$ ,  $p = 0.043$ ). Multivariate linear regression analysis was carried out to determine the statistically significant predictors of serum OPG levels. Including age (standard regression coefficient ( $\beta$ ) = 0.232,  $p = 0.002$ ), cystatin C (standard regression coefficient ( $\beta$ ) = 0.182,  $p = 0.015$ ), FTI (standard regression coefficient ( $\beta$ ) = - 0.178,  $p = 0.012$ ) and E2 (standard regression coefficient ( $\beta$ ) = 0.166,  $p = 0.021$ ) in a regression model showed that age, cystatin C, FTI and E2 were significant predictors of serum OPG levels.

Including cystatin C and E2 in a regression model showed that cystatin C (standard regression coefficient ( $\beta$ ) = 0.345;  $p = 0.002$ ) was the only significant predictor of serum OPG levels in the older men.

There was no statistically significant difference in the group with non-detectable ( $< 0.02$  pmol/L,  $n = 74$ ) sRANKL concentrations as compared to those with detectable ( $\geq 0.02$  pmol/L,  $n = 120$ ) sRANKL concentrations in the studied parameters, except for LS ( $1.190$  ( $0.810$ - $1.892$ ) vs.  $1.143$  ( $0.749$ - $1.855$ )  $\text{gm/cm}^2$ ;  $p = 0.043$ ) and FN ( $0.984$  ( $0.646$ - $1.281$ ) vs.  $0.949$  ( $0.602$ - $1.331$ )  $\text{gm/cm}^2$ ;  $p = 0.049$ ) BMD. As such, LS and FN BMD is significantly lower in those with undetectable sRANKL levels.

There was no difference in OPG, sRANKL and sRANKL/OPG ratio upon comparing the vitamin D sufficient ( $25\text{OHD} \geq 75$  nmol/L) with the vitamin D insufficient ( $25\text{OHD} < 75$  nmol/L) individuals.

### **Examination of DEQAS and HunMen samples**

All samples for the HunMen cohort were reevaluated using the LIAISON<sup>®</sup> DiaSorin CLIA platform for measuring total 25OHD. The timing of these measurements coincided with that of the October 2015 DEQAS survey in order to external quality assurance, using samples 481 to 485. Identical analytic conditions, i.e., reagents with the same LOT number, were insured for both the HunMen sample reanalysis and the DEQAS sample measurements. Finally, the HunMen 25OHD values were corrected using the linear regression equation ( $y = 1,0582x + 6,169$ ) derived from the analysis of relationship between our LIAISON measured total 25OHD values and the DEQAS NIST total target values for all five of the DEQAS samples.

The mean total 25OHD value changed markedly, i.e., from 73 nmol/L, with the original HPLC methodology, to 53 nmol/L as measured by the DiaSorin LIAISON<sup>®</sup> 25OHD total CLIA platform, and following NIST total target value bias standardization to 62 nmol/L. As such, the originally reported prevalence of hypovitaminosis D ( $<75$  nmol/L), as measured by HPLC, increased significantly from 53 to 72%, following standardization of the DiaSorin platform measured values. As reported previously with the HPLC-measured values, on comparing the means in the different seasons, there was a statistically significant difference ( $p < 0.01$ ) between the summer and the other seasons in both the DiaSorin measured and standardized 25OHD values. Furthermore, after standardization, the HunMen values have been found to be at par with values reported by others in Hungary.

## DISCUSSION

### Assessment of factors influencing osteoprotegerin levels

Our results demonstrate that serum OPG levels increase and sRANKL/OPG ratio decreases with age in healthy men over 50 years of age. With regards to the relationship of OPG with age, our findings are in tune with those reported by others. The positive correlation of OPG with age is the only finding showing consensus in the different studies to date. The only exception with this regards is the nonsignificant finding by Oh et al., where the lack of correlation may be due to the limited number of cases studied ( $n = 80$ ) and a relatively lower age maximum of the study population.

With regards to the correlation of OPG with BMD and biochemical markers of bone turnover, as per the results of the studies published to date, statistically significant positive, negative and non-significant was found. Our finding of non-significance only contributes to the need for further studies. Knowing that OPG messenger ribonucleic acid (mRNA) is expressed in a variety of tissues, including lung, kidney and heart, multiple tissues contribute together to circulating OPG, as such measurement of OPG levels in the bone microenvironment is most desirable. Our finding of statistically significant positive correlation between E2 with OPG, along with those reported by Schulz et al. and Indridason et al., supports the finding that E2 increases OPG mRNA steady state levels and protein production in a human estrogen-responsive osteoblastic cell line. Furthermore, Khosla et al. have demonstrated that estrogen treatment increases OPG levels in adult men. Our finding of negative correlation between testosterone and OPG supports two and disagrees with another 2 previous studies. Nonetheless, the negative correlation supports the finding by Khosla et al. where it was demonstrated in vivo that testosterone therapy resulted in lower OPG levels.

Our finding of non-correlation of age with sRANKL is in accordance with that reported earlier. Upon bivariate analysis we found no correlation between sRANKL and BMD, this finding is in agreement with that of Trofimov et al. and Oh et al., but in disagreement with the findings of Stern et al., who reported an inverse association between RANKL and BMD. Nonetheless, we observed statistically higher FN and LS BMD in those with detectable (higher) sRANKL levels, this finding, at least in part, may explain the low risk of non-traumatic fracture in participants in the highest tertile of RANKL in the study by Schett et al. It needs to be pointed out that Schett et al. found no relationship between sRANKL and bone ultrasound data and they did not carry out BMD measurements in their study population. Adding to the controversy is the finding by Jorgensen et al., where they found no difference in BMD between those with detectable versus non-detectable sRANKL

levels. Although the present generation of sRANKL immunoassays has a better lower detection limit than its predecessors, methodology with improved detection limits are most desired. Probably introduction of more sensitive immunoassays may help quantify undetectable sRANKL levels and perhaps may help explore relationships between those with low sRANKL and its known predictors. As far, the one study that studied the relationship of sRANKL/OPG ratio with age found no significance with this regards, in contrast to this our study reports a negative correlation between the ratio and age. This finding certainly needs validation by others.

Although it is suggested that PTH has a suppressive effect on OPG production, the present study did not find any correlation between PTH and OPG. Our results are in agreement with those of Indridason et al. and in contrast to those of Szulc et al. and Kudlacek et al. In contrast to the finding that 1,25 dihydroxyvitamin D stimulates OPG production, in vivo studies in healthy men, including the present study, have not found any correlation between 25OHD, an index of body vitamin D status, and OPG.

In summary, the non-agreement between findings from different studies may be explained, at least partially, by the different assay methodology, in some cases the use of frozen samples more than a decade old, the different recruitment criteria used in the selection of the cohort, the size of the study sample and perhaps the age composition of the cohorts studied.

The role of decreased protein clearance as a potential cause of age-related OPG elevation has been considered in a few previous studies. Mazzioti et al. found a significant positive correlation between creatinine and OPG, and Samelson et al. found that with increasing quartiles of OPG the GFR decreased significantly. This finding is in contrast to the results of Szulc et al., where no correlation was found between OPG and creatinine. Our findings show that there is no correlation between OPG and eGFR, when the MDRD equation is used, but there is a significant negative correlation using the CKD-EPI cystatin C and the CKD creatinine-cystatin C equations. This finding may illustrate the importance of the type of equation used to calculate eGFR. Nonetheless, the combined creatinine-cystatin C equation is considered to perform better than equations based on either of these markers alone. We found a strong positive correlation between OPG and cystatin C. Our positive finding is in contrast to that of Indridason et al., where no such correlation was found. Although the assay methodology used by Indridason et al. is not the one used by us, this difference alone does not explain the difference in the observations of the two studies. Nonetheless, serum OPG has been shown to correlate positively in male patients with chronic renal failure.

Although the study participants were members of a well-defined healthy cohort randomly selected from the population, there are limitations to our study. The HunMen study was not designed primarily for the aim of this manuscript, and only 194 samples were available for serum OPG and sRANKL determination from a total of 206 participants. In addition, we were not in a position to study the effects of bioavailable (free) sex hormones on OPG, instead we used calculated free sex hormone indices to carry out the different statistical analysis. Although the mechanism of the effect, as a renal marker or inhibitor of osteoclastogenesis, of cystatin C on OPG levels cannot be elucidated by the findings of the present study, we have found that it is a significant predictor of serum OPG levels. These findings need verification and further studies are necessary.

The present study demonstrated that in addition to age, the stronger predictor, other adjustable factors such as cystatin C, FTI and E2 were also significant predictors of OPG. Furthermore, the association between cystatin C and OPG was more evident with increased age.

In conclusion, cystatin C is a significant predictor of OPG independently of age, FTI and E2.

### **Standardizing 25-hydroxyvitamin D data from the HunMen study**

Standardization of 25-hydroxyvitamin D (25OHD) values is still a challenge. An overview of the VDSP, which is an international effort coordinated by the Office of Dietary Supplements, NIH, in collaboration with several agencies, institutes and universities, has been provided elsewhere. The initial focus of the VDSP is on standardizing the measurement of 25OHD in national health and nutrition surveys around the world, and there are two basic protocols, one protocol for standardizing current and future 25OHD measurement procedures and another protocol for standardizing 25OHD values from past surveys or studies.

Cashman and colleagues have standardized serum 25OHD values from the Irish National Adult Nutrition Survey conducted in 2008–2010 as suggested by the VDSP protocol executing reanalysis of historic samples with liquid chromatography tandem mass spectrometry (LC-MS/MS). Sempos and colleagues corrected historical vitamin D survey data, where it was assumed that standardization is impossible, using the bias from DEQAS ALTM pertaining to the period when the survey was done. Sarafin and colleagues standardized 25-hydroxyvitamin D values from the Canadian Health Measures Survey. Here, standardization was accomplished by using a two-step procedure. First, serum samples corresponding to the original plasma samples were remeasured by using the currently available immunoassay method. Second, 50 serum samples with known 25OHD values

assigned by the NIST and Ghent reference method laboratories were measured by using the currently available immunoassay method. Lately, Cashman and colleagues applied the VDSP protocol to standardize 25OHD values from four Nordic populations. A specifically selected subset of bio-banked serum samples from each of the studies was reanalyzed for 25OHD by LC-MS/MS and a calibration equation developed between old and new 25OHD data, and this equation was applied to the entire datasets from each study.

The limitations of our study include the use of 6-year-old samples stored at  $-70\text{ }^{\circ}\text{C}$  and an indirect approach. Validation of our method by assessing the accuracy of the calculation on additional serum samples is desirable. Perhaps not a limitation, but our approach would require constant monitoring of the bias from the NIST total target value in each DEQAS survey. As such, after each round of the DEQAS survey, the regression analysis needs to be repeated using the new values and applied to values generated till the results of the upcoming survey are available.

It is not a trivial task in underprivileged settings to follow the VDSP guidelines as such. In Hungary, there is no known laboratory that measures serum 25OHD using the recommended reference measurement procedure in a routine laboratory setting or a research faculty. The financial burden involved in getting historic samples remeasured with LC-MS/MS as such may be too arduous to handle. On the other hand, participation fee for the DEQAS program is quite reasonable and the NIST total target values communicated by the program may come in handy to allow correction of 25OHD values. Our simple approach towards standardizing 25OHD values using the DEQAS survey bias from NIST total target value may encourage participating laboratories to readily correct values at primary result delivery. We are suggesting an alternative to the VDSP requirement of the RMP in settings where the methodology or the financing of analysis is unavailable. We sincerely acknowledge the limitations of our approach but it may suffice where the VDSP protocol cannot be adhered to. Our approach may perhaps complement the VDSP protocol and offer an alternative until commercial assays are developed that yield values that are equal to the NIST concentration for any sample within certain prescribed error limits.

In conclusion, a simple approach readily applicable at the point of conception of the 25OHD values, i.e., at the routine diagnostic laboratory where the measurements are primarily done, could perhaps be a practical solution to the anomaly surrounding non-standardized 25OHD values.



## SUMMARY

In addition to being a key regulator of osteoclastogenesis, the OPG/RANKL/RANK system is reported as being a significant mediator between factors such as estradiol, testosterone, parathyroid hormone, vitamin D, and bone turnover. Data on OPG and RANKL levels in healthy men is accumulating. Nonetheless, based on published data, it may well be generalized that the association between OPG/RANKL and its predictors is lacking consensus. Furthermore, the association of cystatin C with OPG and RANKL has only been limitedly investigated in the healthy, where no significant correlation was found.

We determined serum OPG and sRANKL levels and examined its relationship with cystatin C, age, 25-hydroxyvitamin D, parathyroid hormone, biochemical markers of bone turnover, total 17 $\beta$ -estradiol, total testosterone and L1–L4 and femur neck BMD data available from 194 randomly selected ambulatory men belonging to the HunMen cohort. OPG correlated significantly with age, cystatin C, E2 and FTI. Compared to the middle-aged (age:  $\leq 59$  years,  $n = 98$ ), older men (age  $> 59$  years,  $n = 96$ ) had significantly higher serum OPG and lower sRANKL levels. The older men showed a significant correlation between serum OPG levels and cystatin C and E2. The results of this study demonstrated that in addition to age (which was the stronger predictor), other modifiable factors such as cystatin C, FTI and E2 were also significant predictors of OPG, and that the association between cystatin C and OPG was more evident with increased age (older age group). As such, cystatin C is a significant predictor of OPG independently of age, FTI and E2.

Low 25-hydroxyvitamin D levels are associated with poor skeletal and extra-skeletal health. As such, development of robust healthcare policy to improve vitamin D sufficiency is critical and is primarily based on vitamin D data achieved from measurement of total 25OHD. Nonetheless, vitamin D sufficiency is estimated by determining 25-hydroxyvitamin D concentrations and although there is abundance of data on vitamin D levels, standardization of 25OHD values is still a challenge. We propose standardization by correction of the measured 25OHD values using the linear regression equation derived from the analysis of relationship between total 25OHD values measured by the methodology used by the laboratory and the NIST total target values (TV) reported by the DEQAS for all five of the DEQAS samples in a given survey. We applied our approach to standardize total 25OHD values of the HunMen cohort.

We reanalyzed samples belonging to the HunMen cohort using the automated DiaSorin LIAISON<sup>®</sup> total 25OHD chemiluminescence immunoassay. All samples for the

HunMen cohort were evaluated using the automated DiaSorin LIAISON<sup>®</sup> total 25OHD CLIA. Our laboratory participates in DEQAS, the timing of these measurements coincided with that of the October 2015 DEQAS survey using samples 481 to 485. The HunMen 25OHD values were corrected using the linear regression equation derived from the analysis of relationship between our Liaison measured total 25OHD values and the DEQAS NIST total target values for all five of the DEQAS samples. The mean total 25OHD value changed markedly, i.e., from 73 nmol/L, with the original HPLC methodology, to 53 nmol/L as measured by the DiaSorin LIAISON<sup>®</sup> CLIA platform, and following NIST total target value bias standardization to 62 nmol/L. As such, the originally reported prevalence of hypovitaminosis D (<75 nmol/L), as measured by HPLC, increased significantly from 53 to 72%, following standardization of the DiaSorin platform measured values.

In conclusion, a simple approach readily applicable at the point of conception of the 25OHD values, at the routine diagnostic laboratory where the measurements are primarily done, could perhaps be a practical solution to the anomaly surrounding non-standardized 25OHD values.

## NEW SCIENTIFIC RESULTS AND THEIR CLINICAL RELEVANCE

- Our results suggest that there is a significant positive correlation between age and serum OPG levels, and a negative correlation between age and the sRANKL/OPG ratio in the HunMen cohort.
- In the HunMen cohort, besides age being the strongest predictor of change in OPG levels, cystatin C, FTI and E2 also proved to be statistically significant factors influencing OPG.
- Our results suggest that there is no correlation between OPG and eGFR when using the MDRD formula, but there is a significant correlation between OPG and eGFR when using the CKD-EPI cystatin C and CKD creatinine – cystatin C formula.
- There is a statistically significant increased LS and FN BMD in those individuals who presented with measureable sRANKL levels, i.e.,  $sRANKL \geq 0.02$  pmol/L.
- Contrary to literature findings, which suggest that 1,25(OH)<sub>2</sub>D-vitamin stimulates OPG production, we did not find any correlation between 25OHD and OPG levels.
- Contrary to literature findings, which suggest that PTH has an inhibitory effect on OPG, we did not find any correlation between PTH and OPG levels.
- Our approach to use DEQAS data to formulate a master equation can be readily applied to standardize routine laboratory generated 25OHD values using various methodology in a cost-effective manner.

## LIST OF PUBLICATIONS



**UNIVERSITY of  
DEBRECEN**

**UNIVERSITY AND NATIONAL LIBRARY  
UNIVERSITY OF DEBRECEN**

H-4002 Egyetem tér 1, Debrecen

Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number:  
Subject:

DEENK/325/2018.PL  
PhD Publikációs Lista

Candidate: Éva Jakab

Neptun ID: JEVA

Doctoral School: Kálmán Laki Doctoral School

### List of publications related to the dissertation

1. **Jakab, É.**, Kalina, E., Pethő, Z., Pap, Z., Balogh, Á., Grant, W. B., Bhattoa, H. P.: Standardizing 25-hydroxyvitamin D data from the HunMen cohort.  
*Osteoporosis Int.* 28 (5), 1653-1657, 2017.  
DOI: <http://dx.doi.org/DOI 10.1007/s00198-017-3924-4>  
IF: 3.856
2. **Kulcsár-Jakab, É.**, Pethő, Z., Pap, Z., Kalina, E., Földesi, R., Balogh, Á., Antal-Szalmás, P., Bhattoa, H. P.: Cystatin C as a potential predictor of osteoprotegerin levels in healthy men, a cross-sectional, observational study.  
*BMC Musculoskelet. Disord.* 16 (277), 1-7, 2015.  
DOI: <http://dx.doi.org/10.1186/s12891-015-0684-1>  
IF: 1.684





### List of other publications

3. Pethő, Z., **Kulcsár-Jakab, É.**, Kalina, E., Balogh, Á., Pusztai, A., Gulyás, K., Horváth, Á., Szekanecz, Z., Bhattoa, H. P.: Vitamin D status in men with psoriatic arthritis: a case-control study.  
*Osteoporosis Int.* 26 (7), 1965-1970, 2015.  
DOI: <http://dx.doi.org/10.1007/s00198-015-3069-2>  
IF: 3.445
4. Pethő, Z., **Kulcsár-Jakab, É.**, Onyeka, U., Molnár, Z., Kalina, E., Balogh, Á., Paragh, G., Antal-Szalmás, P., Káplár, M., Bhattoa, H. P.: Az országspecifikus FRAX-algoritmus által meghatározott 10 éves csípő- és major osteoporoticus törések valószínűségének vizsgálata 2-es típusú diabeteses férfiak csoportjában.  
*LAM KID.* 4 (4), 155-161, 2014.

**Total IF of journals (all publications): 8,985**

**Total IF of journals (publications related to the dissertation): 5,54**

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

11 October, 2018



## **ACKNOWLEDGEMENT**

I would like to thank my supervisor, Dr. Harjit Pal Bhattoa, for all his support and constant encouragement.

Furthermore, I would like to express my gratitude towards Prof. Dr. János Kappelmayer for facilitating my activities in the laboratory and to all laboratory personnel who were more than helpful.

I thank Dr. Csernák Zsolt for his support in assuring an asserting background and my immediate colleagues for all their patience and kindness.

Thank you dear Mom and Sisters!